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Morphometric comparisons and novel observations of diurnal and low-light-foraging bees

James B. Dorey[1], Erinn P. Fagan-Jeffries[2], Mark I. Stevens[3,4], Michael P. Schwarz[1]

1 *College of Science and Engineering, Flinders University, GPO Box 2100, SA, 5001, Adelaide, Australia* **2** *Australian Centre for Evolutionary Biology & Biodiversity and School of Biological Sciences, The University of Adelaide, SA, 5000, Adelaide, Australia* **3** *Biological and Earth Sciences, South Australian Museum, GPO Box 234, SA, 5001, Adelaide, Australia* **4** *University of South Australia, Clinical and Health Sciences, SA 5000, Australia*

Corresponding author: James B. Dorey (jbdorey@me.com)

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Abstract

Low-light adapted bees are substantially understudied components of the bee fauna, particularly in Australia. Whilst several species in Australia are thought to be adapted to low-light conditions, explicit records of these taxa actually foraging at twilight or night are absent from the scientific literature. We present the first observations of Australian bees foraging in low-light conditions as well as the first evidence of low-light foraging behaviour in the colletid bee subfamily, Hylaeinae. Using morphometrics of Australian and more broadly-distributed diurnal, facultative low-light and obligate low-light adapted bees, we explore the use of morphological traits to objectively assess possible low-light behaviour and corroborate low-light collection events. Our results show that it is possible to morphologically distinguish between diurnal and low-light adapted bees, and that there is a spectrum of characters that are associated with low light conditions. We use GIS to show that low-light adapted species occur mostly in the tropics, but that some species have subtropical, arid and even temperate distributions. As low-light foraging behaviour in bees is infrequently reported, it appears that low-light foraging behaviour is more common than currently appreciated, highlighting the need for extended bee-sampling periods and more consistent collection data to increase the understanding of this little-understood aspect of bee behaviour.

Keywords

Behaviour, climate, crepuscular, photic niche, morphometrics, nocturnal, pollination, *Reepenia*

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Introduction

Bees play a key role in terrestrial ecosystems, responsible for many pollination services in both crops and native ecosystems (McGregor 1976; Ollerton et al. 2011; Winfree et al. 2011). However, despite the typical representation of bees as organisms associated with daylight foraging, the evolutionary transition to foraging in low-light conditions has occurred multiple times, in some cases with significant subsequent diversification within low-light adapted lineages (Wcislo and Tierney 2009). The suggested evolutionary drivers behind these transitions include reduced competition for resources and escape from enemies such as parasites and predators (reviewed in Wcislo and Tierney (2009)), although these hypotheses currently lack direct evidence from quantitative studies. Regardless of the drivers behind the evolution of the behaviour, bees foraging during low-light conditions can be critically important for particular pollination systems, with some plants specifically adapted to nocturnal bee visitors (e.g., Cordeiro et al. (2017)).

Low-light adapted bees can be described as matinal (foraging in the pre-dawn twilight), vespertine (post-sunset twilight foragers), crepuscular (foraging both pre-dawn and post-sunset), or nocturnal (foraging at night). Bees active in low-light conditions can also be classified as obligate (restricted to foraging in low-light conditions) or facultative (capable of, but not restricted to, foraging in low-light conditions). Whilst some species have been the focus of studies which recorded light levels and flight times, for example *Xylocopa tranquebarica* (Fabricius, 1804) (Apidae) from Southeast Asia (Burgett and Sukumalanand 2000; Somanathan et al. 2008) and *Megalopta genalis* Meade-Waldo, 1916 (Halictidae) from Panama (Kelber et al. 2005), many species are inferred to be low-light adapted only because they have been collected at light-traps (e.g., Wolda and Roubik (1986)) or by anecdotal evidence. Several morphological characters have also been used as evidence that particular species are adapted to foraging in low-light conditions, including enlarged ocelli and compound eyes (Kerfoot 1967a; Warrant 2008), enlarged ommatidia, and reduced pigmentation. However, not all bees that have been inferred to be obligate twilight or nocturnal foragers have such phenotypes (Wcislo and Tierney 2009). While morphological characters have been used to loosely support hypotheses of species being adapted to low-light conditions, there are no formal statistical analyses of how these characters vary across species known to be diurnal, obligate low-light, and facultative low-light foragers.

Current data suggest that bees foraging in low-light conditions are more common in tropical forests and deserts (Wcislo and Tierney 2009), but there is a considerable lack of data on both facultative and obligate low-light foraging bees in many parts of the world. In Australia, there are several species from central and northern regions that have morphological characteristics that suggest adaptation to low-light and, on this basis, have been hypothesised to be adapted to foraging in either twilight or darkness. The halictine bee *Homalictus rowlandi* (Cockerell, 1910) (Halictidae) from the east coast of Australia, and the widely-distributed *Lasioglossum ochroma* Walker, 1995 (Halictidae) are suggested to be adapted to low-light conditions due to reduced pigmentation (Dorey 2018; Houston 2018). Additionally, there are several species with enlarged

ocelli, including *Meroglossa canaliculata* Smith, 1853 (Colletidae), *M. gemmata* Houston, 1975 and *M. ocellata* Michener, 1965 from northern Australia, and *Reepenia bituberculata* (Smith, 1853) (Halictidae) from northern Queensland (Michener 2007; Houston 2018). The three *Meroglossa* species currently thought to be adapted to low-light are part of the *Meroglossa impressifrons* species-group as designated by Houston (1975), a group of six species divided into two subgroups: those with 'normal' ocelli (*M. impressifrons*, *M. punctata* Rayment, 1935 and *M. soror* Perkins, 1912), and those with enlarged ocelli (*M. canaliculata*, *M. gemmata* and *M. ocellata*). There have been several collections of *M. gemmata* at light traps (see https://flic.kr/p/5wpksW and https://flic.kr/p/5wk2eP for examples; pers. comm. Bernhard Jacobi) but with no associated plant records or foraging observations. *Reepenia bituberculata* is the only described species of the genus that is recorded in Australia, and whilst there have been previous observations of nocturnal behaviour (S.M. Tierney pers. comm., cited in Freelance et al. (2019)) and records of the species being collected at light traps (PaDIL registration number 57995: http://www.padil.gov.au/pollinators/pest/specimens/138403) there is no formal documentation of the species actually foraging in low-light conditions.

Generally, bees are collected in the field by researchers targeting flowering plants during daylight hours, when most species are known to be active. There are also possible difficulties in observing the bees in low-light environments without using light that could interfere with their behaviour. Observations of bees foraging in low-light conditions are therefore rare, and often occur inadvertently; identifying the morphological and climatic characters associated with these behaviours will aid in their identification and documentation. Hence, our research aims to fill several knowledge-gaps. Firstly, we present observations of low-light foraging of several species: *R. bituberculata*, and three *Meroglossa* species (*M. eucalypti* Cockerell, 1910, *M. gemmata* and *M. impressifrons penetrata* (Smith, 1879)) belonging to the *impressifrons* and *eucalypti* species-groups and hereafter referred to as *Meroglossa* spp. Secondly, we use these species, and other Australian and more broadly-distributed diurnal, facultative and obligate low-light-adapted species, to statistically analyse morphological traits and infer morphological adaptations to low-light behaviours in bees. Finally, we examine data from the Atlas of Living Australia (ALA 2020) and the Global Biodiversity Information Facility (GBIF.org 2020a) to explore climate-related patterns in the distributions of these species.

Methods

Observation periods and locations

Initial observations for *Reepenia bituberculata* were undertaken from the 16–18 Nov. 2019 in the Daintree rainforest near Thornton Beach from the cultivated palm tree, *Dypsis lutescens* (H.Wendl.) Beentje & J.Dransf. (Arecaceae; Suppl. material 1: Tables S1, S2). This site was on the boundary of rainforest with a thick canopy and a largely-cleared caravan park, with some distant man-made light sources. Observations

were made on primarily clear evenings. These observations were undertaken during a waning gibbous moon. Combining sporadic observation periods over the three days, several inflorescences on a single *D. lutescens* were observed between 0500 h (36 mins before sunrise) and 0835 h (181 mins after sunrise) in the morning, and between 1600 h (149 mins before sunset) and 1935 h (126 mins after sunset) in the evening. Intermittent observations of the palm extended to as late as 1038 h in the morning and 2300 h in the evening. Activity levels for *R. bituberculata* were quantitatively recorded on the morning of 17 Nov. 2019, where the number of females present on the flowers of one palm were counted at the start of every five-minute period between 0500 h and 0610 h. Notes on foraging activity and the numbers of both males and females were also qualitatively recorded sporadically throughout the observation periods.

Further observations for *R. bituberculata* were undertaken between the 20–21 Feb. 2020 in Cairns from the native palm, *Licuala ramsayi* (F. Muell.) Domin (Arecaceae; Suppl. material 1: Tables S1, S2). This site was in a city with artificial light sources (e.g., lamp-posts and houses) present. The recorded observations were undertaken during a waning crescent moon. General sweeps were undertaken sporadically (approximately hourly) between 0800 h and 2300 h.

Observations and collections of *Meroglossa* species were made at a crepuscular collection event near Laura, Queensland on *Melaleuca leucadendra* (L.) L. on the 18 Nov. 2019 (Myrtaceae; Suppl. material 1: Tables S1, S2). This site was at a dry river bed, without a complete canopy and observations were made on a clear evening. The recorded observations were undertaken during a waning gibbous moon. The observation period began at 1858 h (25 mins after sunset) and continued until 1959 h (86 mins after sunset).

Morphometric analyses

Taxon selection and identification

Specimens for imaging, identification and morphometric analyses were amalgamated from recent collections by J.B. Dorey and E.P. Fagan-Jeffries. Representatives of obligate and facultative low-light species are deposited at the South Australian Museum, Adelaide (SAMA) (Suppl. material 1: Tables S1, S3). Other Australian specimens form part of an image-reference collection maintained by J.B. Dorey (Suppl. material 1: Table S3). Specimens were identified to genus or the lowest possible taxonomic rank using available taxonomic keys (Exley 1974; Houston 1975; Walker 1995; Leys 2000; Batley and Houston 2012; Dollin et al. 2015; Rocha-Filho 2016; Leijs et al. 2017; Smith 2018; Leijs et al. 2020). Australian bee species were named according to the Australian Faunal Directory (AFD 2019). Hence, *Homalictus* was afforded generic status (i.e. not referred to as a subgenus of *Lasioglossum*) as has been done in most recent literature (e.g. (Bernhardt et al. 2019; Dorey et al. 2019; Hall et al. 2020; Neave et al. 2020)). Thirteen additional non-Australian bee specimen images were sourced from the Natural History Museum UK (Natural History Museum 2014a) in order to provide broader taxonomical and geographical low-light representation. Species included in the analyses were chosen based on availability of specimens and size-calibrated images.

The final dataset included 75 specimens from 68 species (Suppl. material 1: Table S3). Of these specimens, 47 were female (diurnal: 32, facultative low-light: three and obligate low-light: 12) and 28 were male (diurnal: 25, facultative low-light: one, obligate low-light: three) (Suppl. material 1: Table S3). References for the observational data leading to species being classified as low-light bees are provided in the Data Resources section and Suppl. material 1: Table S3. Specimens spanning all Australian bee families were included (Apidae: 19, Colletidae: 31, Halictidae: 16, Megachilidae: eight and Stenotritidae: one) with both Australian (62) and extralimital (13) specimens included (Table 1).

Imaging, trait selection and measurement

Morphological traits were measured using photographs of known scale. Images of the Australian species were taken using either a Canon EOS 5D mk iv or Canon EOS 5DSR camera with a Canon MPE-65 or Canon EF 100–400mm IS L II with a Nikon 4× or 10× plan achromat microscope objective. Morphology measurements were taken using Adobe Photoshop version 21.1.0.

We measured 13 morphological traits and, from these, we derived seven (ratio or product) traits that might be associated with diurnal or low-light foraging (see Fig. 1; Suppl. material 1: Table S3). Traits relating to vision (e.g. median ocellus distance and eye area) and body size (e.g. mesosoma length and intertegular distance) were chosen as they have been suggested as important traits low-light traits (Wcislo and Tierney 2009) and are easily measured even on relatively low-resolution images.

Statistical analyses

We took two multivariate approaches for analysing the morphological data and these were done separately for males and females. Firstly, we conducted a principal components analysis (PCA) using seven of 13 measured morphological traits and seven derived traits (Fig. 1; Suppl. material 1: Table S3). Head width, head depth, mesosoma length and intertegular distance were excluded from analyses because they are included as contributing to derived traits (Fig. 1). Similarly, the ommatidial areas were excluded because it is essentially the inverse of ommatidial density (Fig. 1). The derived ratio measurements are included in analyses because we regarded them as meaningful func-

Table 1. The number of diurnal, facultative and low-light species examined by family and the number of Australian and extralimital species examined by family. *There are no available behavioural data to suggest that these species are other than diurnal.

Family	Diurnal*	Facultative	Obligate	Australian	Extralimital
Apidae	15	0	3	15	3
Colletidae	17	3	7	21	6
Halictidae	10	0	4	10	4
Megachilidae	8	0	0	8	0
Stenotritidae	1	0	0	1	0
Total	51	3	14	55	13

Direct measurements	Derived measurements
Median ocellus width (MOW; mm)	MOW to OD ratio (MOW:OD)
Inter-ocellar distance (IOD; mm)	Eye area to head area ratio front
Ocello-ocular distances (OD; mm)	Eye area to head area ratio side
Eye area front (EAF; mm^2)	Ommatidia density front (ommatidia/mm^2)
Total face area (TFA; mm^2)	Ommatidia density side (ommatidia/mm^2)
Eye area side (EAS; mm^2)	Head width x head depth (HWxHD; mm^2)
Area of head side (HAS; mm^2)	Mesosoma length x intertegular distance (MLxID; mm^2)
Head width (HW; mm)	
Head depth (HD; mm)	
Mesosoma length (ML; mm)	
Intertegular distance (ID; mm)	
Area of 7 ommatidia front (7c measured twice; mm^2)	
Area of 7 ommatidia side (7c measured twice; mm^2)	

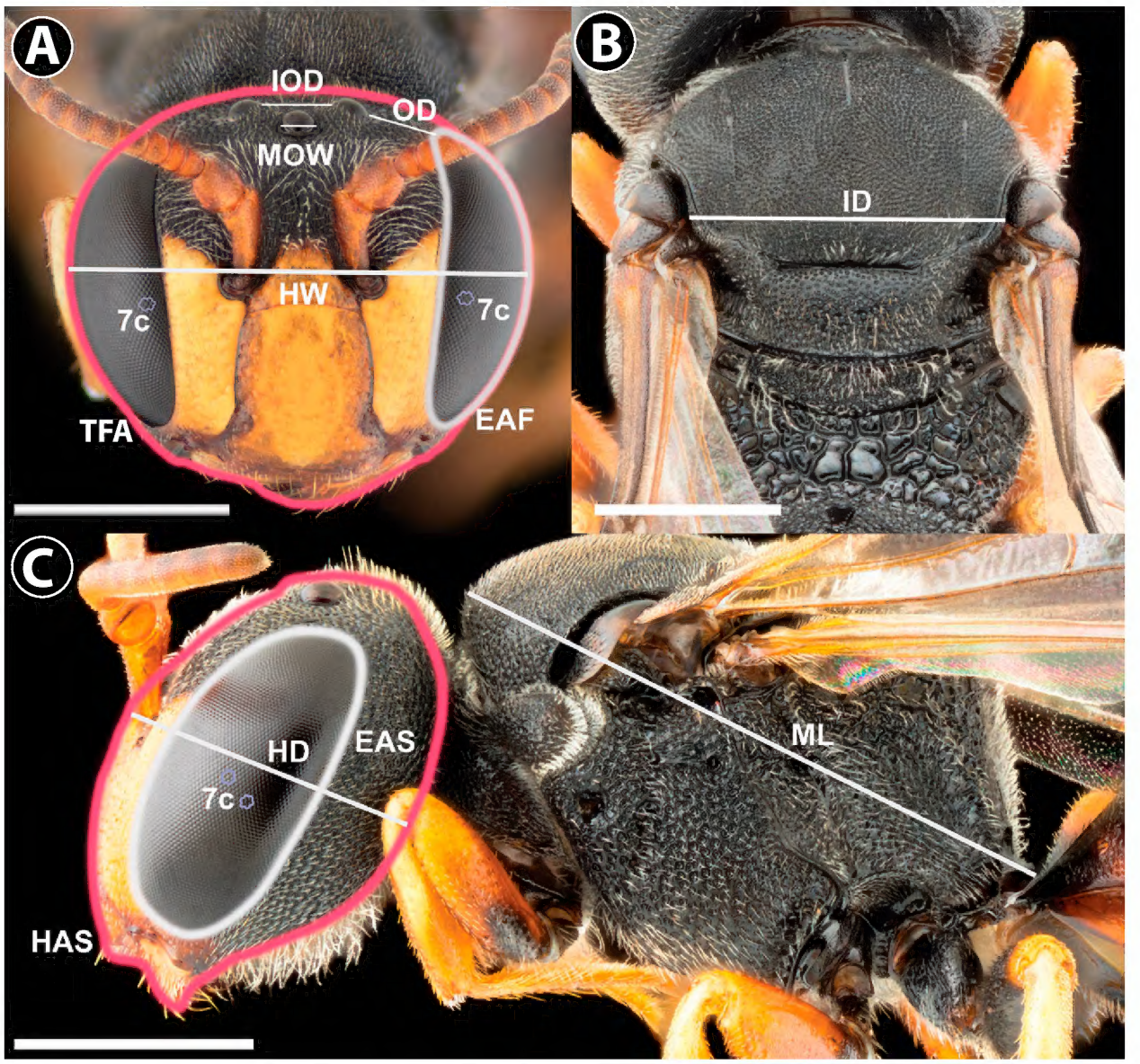

Figure 1. Morphology measures taken from **A** frontal; inter-ocellar distance (IOD), ocello-ocular distance (OD), median ocellus width (MOW), head width (HW), the area of seven ommatidia twice (7c), eye area front times two (EAF), total face area (TFA) **B** above; intertegular distance (ID) and **C** laterally; head area side (HAS), the area of seven ommatidia twice (7c), head depth (HD), eye area (EAS) and mesosoma length (ML). Bars are 1 mm in length. Italicised direct measures are those that were not included in the final analyses. Example species is the diurnal *Hylaeus* (*Hylaeteron*) *hemirhodus* (Colletidae: Hylaeinae).

tional traits (see results). Principal component scores were then used in a discriminant analysis to determine whether these scores could be used to reliably assign species into diurnal and low-light categories. PCA was conducted using SPSS version 25 for Windows (IBM Corp. 2017) with no rotation of factors and retaining regression scores for components with eigenvalues greater than 1.0. PCA plots and normal probability ellipses were created in the R statistics platform version 3.6.2 (R Development Core Team 2019) using the packages *ggbiplot* version 0.55 (Vu 2011) and *ggplot2* version 3.3.0.9 (Wickham 2016) while scree plots and heatmaps were created using *ComplexHeatmap* version 2.3.4 (Gu et al. 2016). Discriminant analyses were conducted using SPSS. For discriminant analyses we used a stepwise Wilks procedure with a default criterion for entry of factors of F(entry) = 3.84 and a removal criterion of F(removal) = 2.71. Discriminant analysis was based only on two groups: species that were scored as diurnal or else low-light, but the discriminant functions were later used to predict group membership for species with facultative photic niches. Photic niche was assigned based on confirmed behavioural groups (i.e. not inferred from PCA results; Suppl. material 1: Table S3). Discriminant analyses did not assume that the number of species was equivalent for each group, and the discriminant function was used to predict group membership which was then compared to actual membership to provide a measure of how effective the discriminant function was.

GIS analyses

To determine the primary climate zone of each species, Darwin core data were downloaded from both the Atlas of Living Australia and the Global Biodiversity Information Facility (ALA 2020; GBIF.org 2020a); a list of GBIF data sources used in the study are provided in the references section. These data were filtered to exclude points without reliable spatial information or potentially uncertain identifications (e.g., human observation or images). Data points were then overlaid with a 1 km resolution worldwide Köppen-Geiger climate classification map (Beck et al. 2018) in QGIS version 3.10.4 (QGIS Development Team 2020). Climate classifications were simplified to the broad categories of arid, cold, subtropical, tropical and temperate. Histograms were created using R graphics packages and the most frequently inhabited climate zone recorded for each species. Climate-zone data were then analysed with the R stats package using a Pearson's Chi-squared and Fisher's Exact tests with significances based on 10,000 Monte Carlo simulations.

Results

Reepenia bituberculata observations

In the Daintree rainforest, *Reepenia bituberculata* was observed on the flowers of *Dypsis lutescens* between 0500 h (the start of the observation period) and 0550 h (36 mins before and 15 mins after sunrise) in the morning, and bees were not observed on flow-

ers for the rest of the morning observation period which extended to 1038 h (Fig. 2). Morning activity of *R. bituberculata* was greatest in the ten minutes either side of civil start (sun 6° below horizon), with the number of foragers decreasing towards and after sunrise (Fig. 2). Afternoon foraging was observed between 1607 h (near the start of the observational period) and 1900 h (82 mins before and 31 mins after sunset, respectively), after which activity ceased and *R. bituberculata* was not observed foraging during the rest of the observation period (until 2300 h) (Fig. 2). Whilst the numbers of foragers were not quantitatively recorded during the evening observation period, qualitatively it appeared that *R. bituberculata* was numerous between 1750 h and 1834 h, and in similar numbers to peak morning activity (Fig. 3). Male *R. bituberculata* were seen to patrol very quickly around the flowers while the females were foraging for nectar and pollen. Despite apparent proximity to females as they foraged, no attempted matings were observed. The only other bee species observed foraging on the flowers in twilight periods was *Homalictus atrus* Walker, 1986, which was first observed ten minutes before sunrise. In the Daintree rainforest, *R. bituberculata* was already foraging at the start of both the morning and afternoon observation periods, but we do not have records prior to these periods. Similarly, no observations were conducted during the middle of the day, so the possibility of *R. bituberculata* foraging outside the observation periods cannot be excluded, but no bees were seen to forage in the observation period of 0550 h to 1038 h.

In Cairns, *R. bituberculata* was less abundant and was only caught between 1830 h and 1900 h (17 min before and 13 min after sunset). Sampling effort throughout the day was haphazard, but greater than that in the Daintree rainforest, and despite hundreds of other bees caught on *Licuala ramsayi* throughout the day (223 specimens with more released; da Silva and Dorey 2020, unpubl. data). Specimens included species of *Braunsapis* Michener, 1969 (Apidae), *Homalictus* Cockerell, 1919, *Palaeorhiza* Perkins, 1908 (Colletidae) and *Hylaeus* Fabricius, 1793 (Colletidae), but there were no

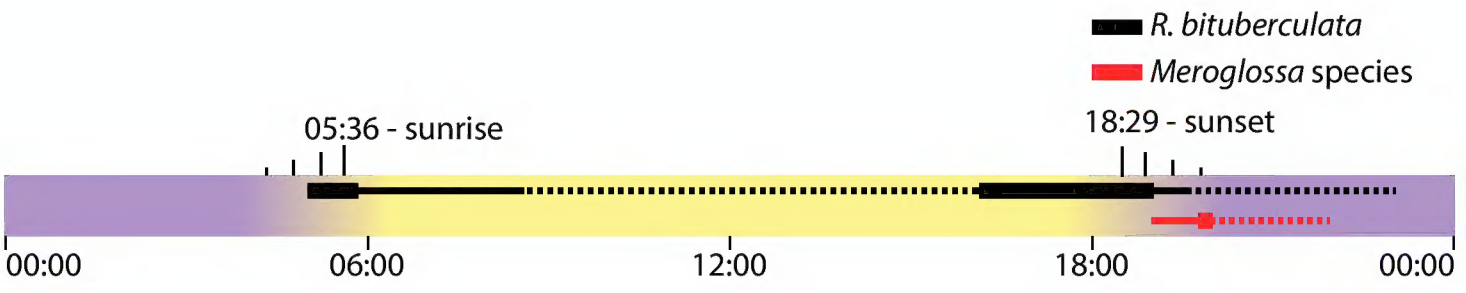

Figure 2. Foraging observations of *Reepenia bituberculata* and *Meroglossa* species. The bar shows night (purple) and day (yellow) with sunrise and sunset indicated above by longest vertical lines and civil (sun 6° below horizon), nautical (sun 12° below horizon) and astronomical (sun 18° below horizon) start and end shown by the lines decreasing in height to the left and right of sunrise and sunset, respectively. Numbers along the bottom of the bar indicate times of day. Thick horizontal black and red lines show when *R. bituberculata* and *Meroglossa* spp., respectively, were collected or observed to be active. Thinner solid lines show frequent observation periods (observed at least every 10 mins) when no bees were observed or caught on flowers, while dotted lines indicate sporadic observation periods (observed every hour or more) when no bees were observed or caught on flowers.

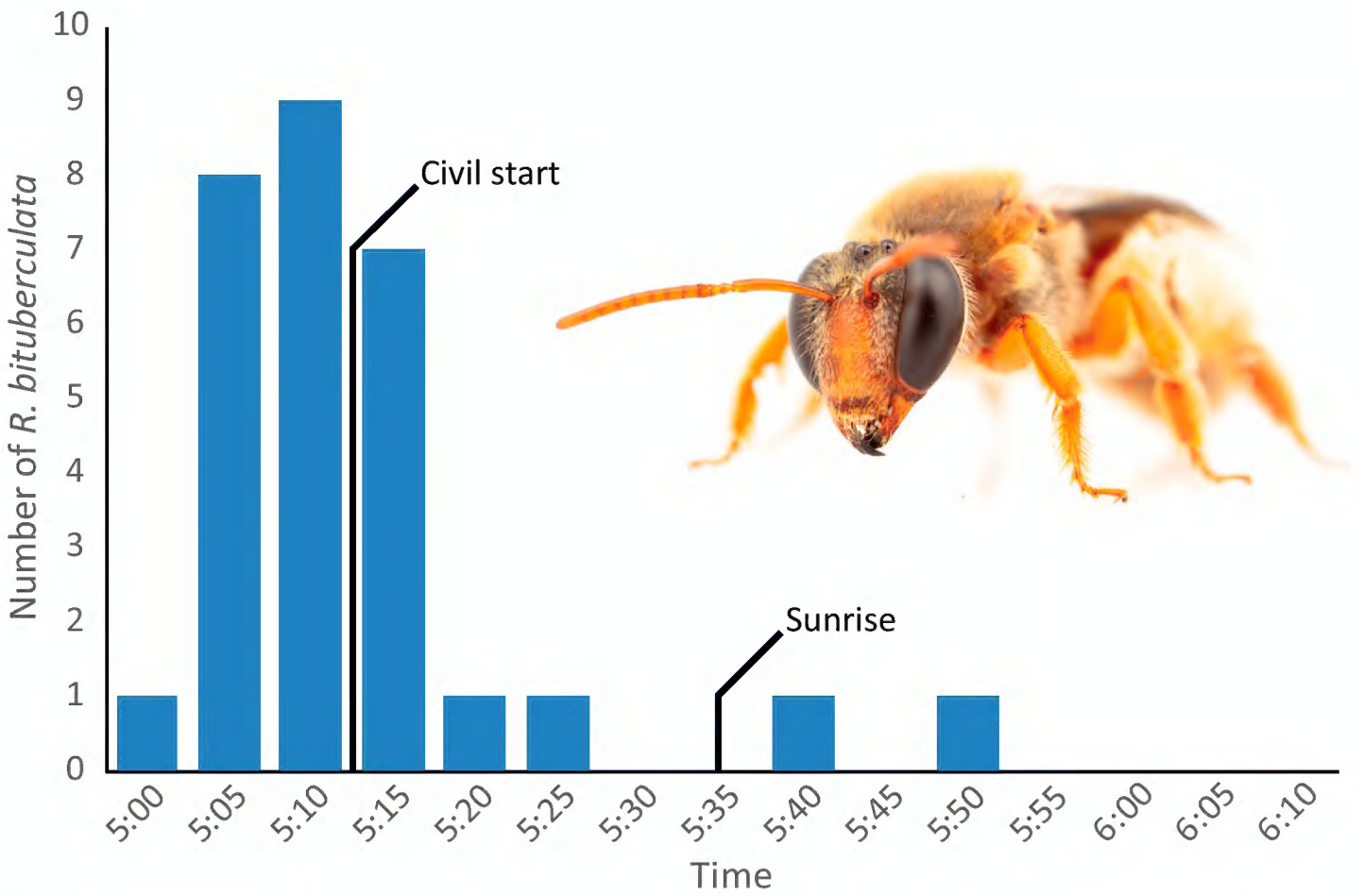

Figure 3. Number of *Reepenia bituberculata* on *Dypsis lutescens* palm flowers on the observed tree at the start of every five minute period on the morning of 17 Nov 2019. Civil start and sunrise are indicated by black vertical lines. Bee image is a female *R. bituberculata*.

diurnal collections of *R. bituberculata*, supporting the hypothesis that *R. bituberculata* is inactive throughout much of the day. Across both sites, foraging was only observed in the early morning twilight, and in the late afternoon continuing into the post-sunset twilight (Fig. 2).

Meroglossa spp. observations

At the *Melaleuca leucadendra* collection event in Laura, bee species from several genera were caught after civil end (sun 6° below horizon), including: *Amegilla* Friese, 1897 (Apidae; 1 sp.), *Braunsapis* (1 sp.), *Euryglossina* Cockerell, 1910 (Colletidae; at least 4 spp.), *Homalictus* (1 sp.), *Hylaeus* (3 spp.), *Meroglossa* (3 spp.), *Pachyprosopis* Perkins, 1908 (Colletidae; 1 sp.) and *Tetragonula* Moure, 1961 (Apidae; 1 sp.) (Suppl. material 1: Table S1). *Meroglossa* species were caught only towards the end of the collection event, where three species (*M. eucalypti*, one specimen; *M. gemmata*, one specimen; and *M. impressifrons penetrata*, two specimens) were collected in quick succession between 1945 h and 1959 h (72 to 86 mins after sunset, and either side of astronomical end (sun 18° below horizon), which occurred at 1950 h) (Suppl. material 1: Table S1). When the three *Meroglossa* species were collected, the host plant could only be perceived as a silhouette. For this site, we have no information available on the foraging activity pre-sunrise or post-moonrise (Fig. 2).

Atlas of Living Australia data and other collections

As of Oct. 2019, there were a combined total of 147 records on ALA for the bees *R. bituberculata* (N = 30), *M. eucalypti* (N = 27), *M. gemmata* (N = 47) and *M. impressifrons* (N = 43; combining both subspecies *M. impressifrons penetrata* and *M. impressifrons impressifrons*). There were only three records of actively foraging bees with time data, one *M. impressifrons impressifrons* (Tobias Smith; 0830 h, 28 Sep. 2018, on *Melaleuca* sp.) and two *M. impressifrons penetrata* (Tobias Smith; 1800 h, 24 Jan. 2019, on *Eucalyptus crebra* F. Muell. (Myrtaceae) and Tony Eales; 1440 h, 18 Sep. 2019, on *Melaleuca* sp.). Other collections of *M. impressifrons penetrata* specimens were made in Ravenshoe, QLD (1548 h, 05 Feb. 2019) and Mareeba, QLD (1120 h, 09 Feb. 2019). All of these observations were made during daylight hours, suggesting that of the *Meroglossa* species collected after sunset in Laura, at least *M. impressifrons penetrata* is not an obligate low-light forager, and will also forage diurnally.

Morphological results

Our PCA analyses returned three principal components (PC) with eigenvalues > 1 that jointly explained ~86% of the total variation for both sexes (Suppl. material 1: Table S4). A scree plot of eigenvalues (Suppl. material 2: Fig. S1) indicates a sharp decline moving from PC1 to PC2 and then lower declines moving to subsequent components, suggesting that only a few underlying factors explain most of the variation in our data. Plots of factor scores from PC1 (explaining 59% of total variation in females, and 63% of variation in males), PC2 (females: 19%, males: 17%) indicate substantial separation between diurnal and low-light foraging bees in both females and males (Fig. 4; Suppl. material 3: Fig. S2). Plots of factor scores from PC1, PC2 and PC3 (explaining 8% of total variation in females and males) completely separate diurnal and low-light foraging bees in both females and males (Suppl. material 4: Fig. S3 and Suppl. material 5: Fig. S4). The loading vectors suggest that for males and females, PC1 is strongly associated with body, head and eye size as well as ommatidial density and PC2 is most strongly related to compound eye to head area ratios, median ocellar width to ocello-ocular distance ratios (**MOW:OD**) and **OD** (Fig. 4; Suppl. material 2: Fig. S1, Suppl. material 3: Fig. S2). Although **OD** is a direct measure, the other variables are all ratios involving head and eye size and might therefore indicate a role for eye size relative to body size. Both of these principal components contribute strongly to the separation of diurnal from low-light species. While the addition of PC3 allows complete separation of behaviours, the factor loadings are more difficult to interpret. However for both sexes, PC3 appears to be associated with visual characters (e.g. eye to head ratios and **MOW:OD** for males and **IOD**, **OD**, eye to head ratio side and ommatidial density front for females; Suppl. material 2: Fig. S1).

Generally, diurnal species had denser ommatidia and larger ocellocular distances (**OD**), while low-light species had larger **MOW:OD** (e.g., Fig. 5) and eye to head area

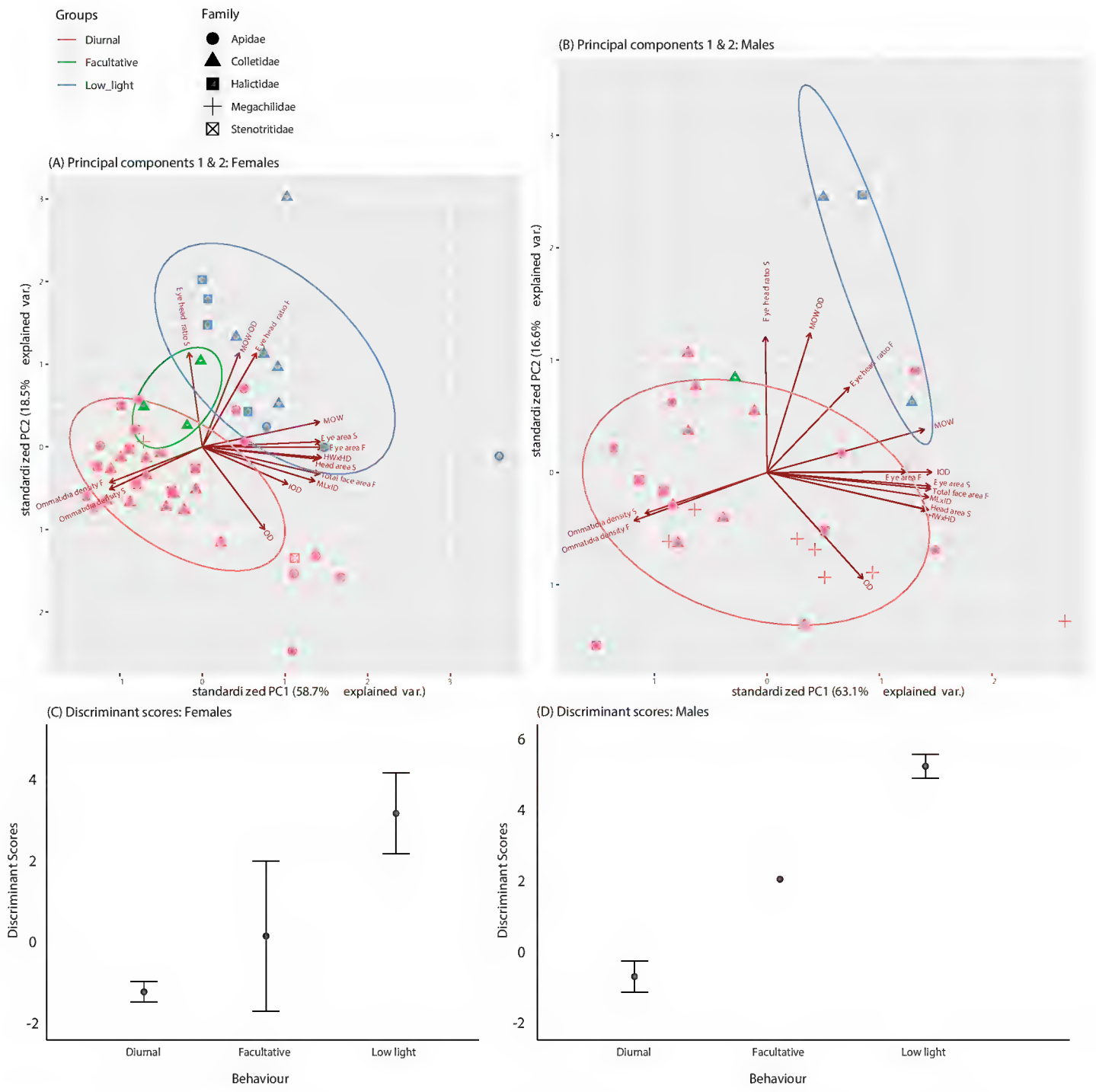

Figure 4. Principal components one and two of **A** female and **B** male bees where symbols indicate family (Apidae: circles, Colletidae: triangles, Halictidae: squares, Megachilidae: crosses and Stenotritidae crossed-squares) and colour indicates known foraging behaviour (diurnal: red, facultative low-light: green and obligate low-light: blue). Measurements are defined in Fig. 1. Plots A and B indicate relative factor loadings for the morphological traits as vectors from the centroid. Discriminant scores for both **C** female and **D** male analyses for each behavioural group showing means and 95% confidence intervals.

ratios on the front and side (Fig. 4). With the exception of **OD**, the above variables are all ratios derived from directly measured traits. Most of the remaining direct measures, excepting median ocellus width, relate to body size and, generally, low-light adapted bees tended to be larger (Fig. 4). Although we had small sample sizes for facultative bees, these species and those associated with them (see below) tended to have relatively large eye to head ratios, and **MOW:OD** ratios (Fig. 4). Additionally, compared to low-light adapted species, facultative species had denser ommatidia and were generally

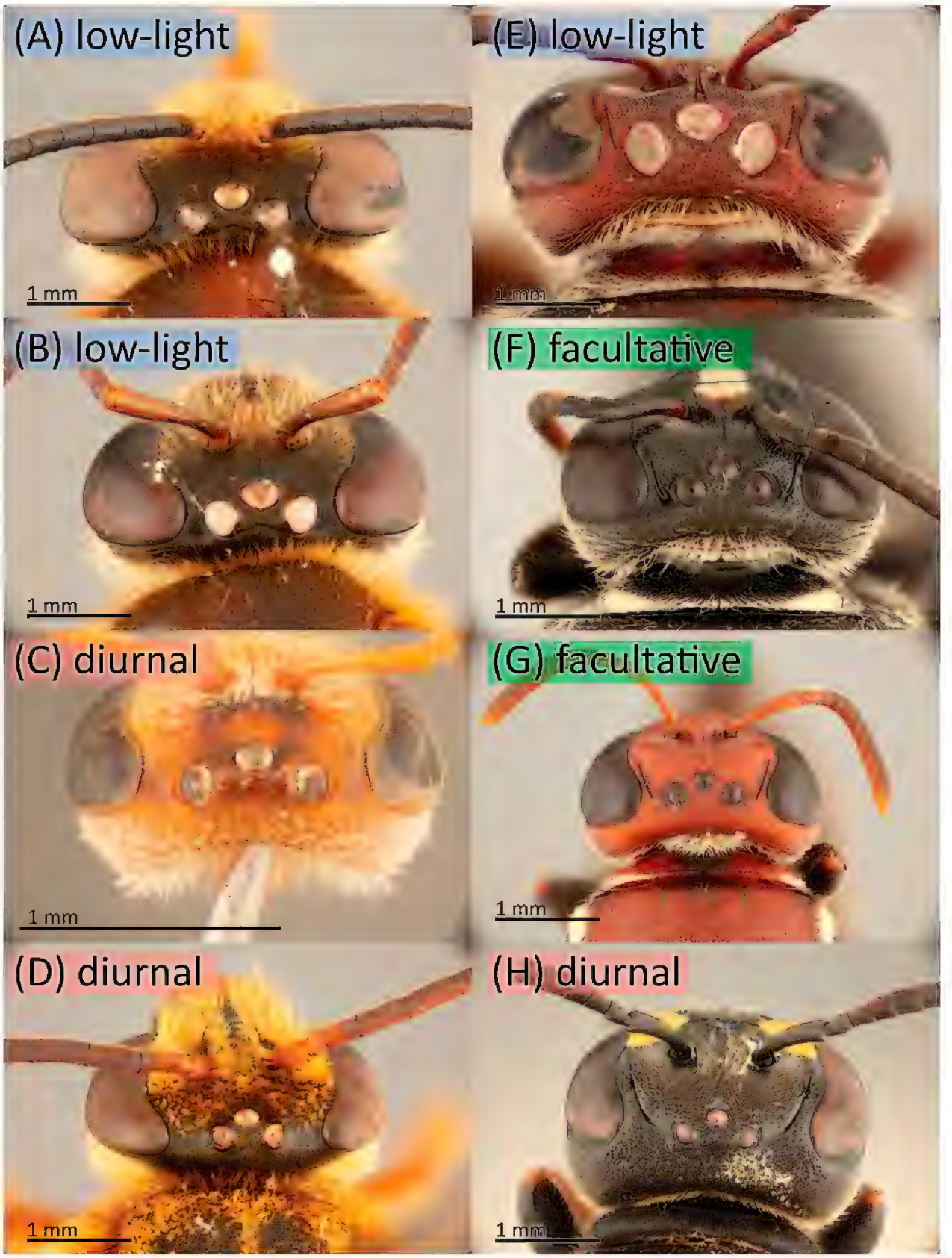

Figure 5. Dorsal head of some included species and related species for comparison of ocelli size **A** male and **B** female *Reepenia bituberculata* (Halictidae: Nomiinae) collected in low-light conditions **C** *Lasioglossum* (*Chilalictus*) *ochroma* (Halictidae: Halictinae), a species that has been hypothesised to be adapted to low-light conditions due to loss of pigment **D** *Mellitidia tomentifera* (Halictidae: Nomiinae), a bee in the same subfamily as *Reepenia* without enlarged ocelli **E** *Meroglossa gemmata* (Colletidae: Hylaeinae) **F** *M. impressifrons penetrata* (Colletidae: Hylaeinae) **G** *M. eucalypti* (Colletidae: Hylaeinae) **H** *Hylaeus* (*Analastoroides*) *foveatus* (Colletidae: Hylaeinae) a bee in the same subfamily as *Meroglossa* that lacks enlarged ocelli. *Meroglossa gemmata* has enlarged ocelli, consistent with adaptation to low-light conditions, comparable in relative size to *R. bituberculata*. The other two *Meroglossa* spp. (*M. impressifrons penetrata* and *M. eucalypti*) do not show such extreme morphological features compared to other members of the genus examined by Houston (1975), but are facultative low-light adapted. When compared to representatives from their respective subfamilies both *Meroglossa* (Hylaeinae) and *Reepenia* (Nomiinae) have relatively enlarged ocelli. Colours refer to those for each behavioural state in Fig. 4.

smaller (Fig. 4). Together, plots of factor scores for PC3 and PC4 were less marked for distinguishing photic niches (Suppl. material 3: Fig. S2C, D).

Our stepwise discriminant analyses retained all three principal components for both females and males and the standardized canonical coefficients are given in Suppl. material 1: Table S5. The discriminant functions were able to correctly predict photic niche membership for 100% of both females and males where diurnal or low-light niches were unambiguous, suggesting that membership of these two niches is accompanied by very clear morphological traits.

Lastly, we calculated discriminant function scores, along with 95% confidence limits, for all females and males in our data set, including those species where photic niche was facultative. These results show clear separation between diurnal and low-light behaviours, with facultative species intermediate between the two behavioural groups for both sexes (Fig. 4C, D).

Known facultative bees were only from the Colletidae and obligate low-light foraging bees included species from the families Apidae, Colletidae and Halictidae. Several species currently thought to be only diurnally active fall out near known facultative-low-light species, including apids, colletids, halictids and a single megachilid species (Table 2). Several 'diurnal' *Amegilla* species fell out near obligate low-light species, where PC2 > 0 (e.g., near the known crepuscular species *Megalopta genalis*, Fig. 4; Suppl. material 3: Fig. S2). In particular, three *Amegilla* species, *A.* (*Zonamegilla*) *cingulata* (Fabricius, 1775), *A.* (*Z.*) *adelaidae* (Cockerell, 1905) and *A.* (*Notomegilla*) *aeruginosa* (Smith, 1854) as well as the kleptoparasites of *Amegilla*, *Thyreus nitidulus* (Fabricius, 1804) and *T. caeruleopunctatus* (Blanchard, 1840) (Apidae; Table 2) (Houston 2018). *Amegilla* species are known to forage or be active throughout the day and in the early morning or evening (e.g., Suppl. material 1: Tables S1, S6). All of these species can be regarded as facultative-associated as they are all known to forage diurnally, and therefore cannot be obligate low-light foragers.

Table 2. The 'diurnal' bee species that, according to our PCA (Fig. 4), are associated with facultative low-light foraging species. Table is broken up by sex and family. The climatic zone in which is species most frequently has been collected is indicated in parentheses where A, S, T, and Te refer to arid, subtropical, tropical and temperate respectively (Suppl. material 6, Fig. S5). Most species did not include both male and female representatives – this was based on availability of specimens and size-calibratable images. The only species in the table with both sexes examined is *E. xanthoclypeata*.

Family	Males	Females
Apidae	*Austroplebeia cassiae* (T)	*Nomada australensis* (T)
	Exoneura (*Brevineura*) c.f. *xanthoclypeata* (Te)	*Exoneurella eremophila* (A)
	Thyreus nitidulus (T)	*Amegilla* (*Zonamegilla*) *adelaidae* (T)
	Thyreus caeruleopunctatus (A)	*Amegilla* (*Notomegilla*) *Aeruginosa* (T)
		Amegilla (*Zonamegilla*) *cingulata* (T)
Colletidae	*Hemirhiza melliceps* (Te)	
	Palaeorhiza (*Callorhiza*) *turneriana* (T)	
	Pharohylaeus lactiferus (S)	
	Palaeorhiza sp.	
Halictidae		*Patellapis* (*Pachyhalictus*) *stirlingi* (T)
		Homalictus (*Homalictus*) *atrus* (T)
Megachilidae		*Megachile* sp.

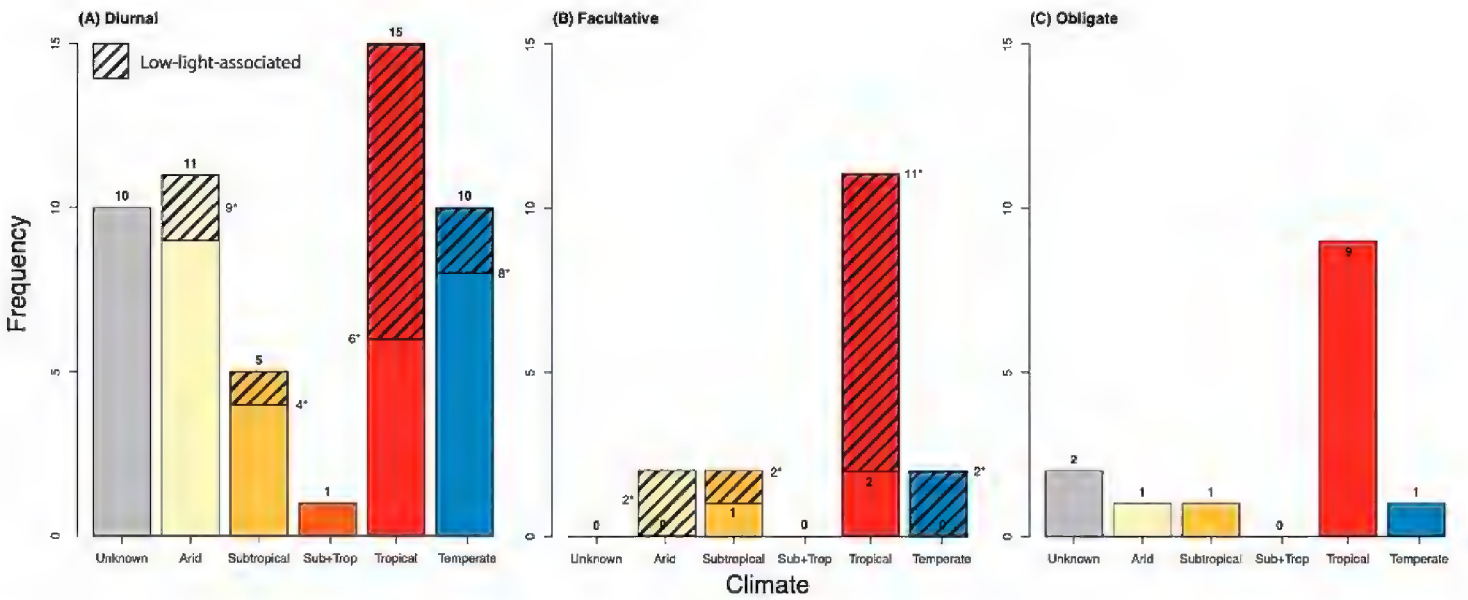

Figure 6. Stacked frequency histograms of the diurnal **A** facultative low-light **B** and obligate low-light **C** bee species by the climate in which they were most frequently collected. Unknowns represent the species that could not be identified reliably to species or, in the case of two low-light species, those with no reliable coordinates (Suppl. material 6: Fig. S5). Patterned bars represent 'diurnal' species that were associated with facultative species, according to our PCA analyses (Fig. 4, Table 2); these species are indicated twice once in (**A**) and once in (**B**). Bolded numbers indicate the behavioural assignment of species without reassigning behavioural state according to PCA results (Table 1), numbers with asterisks indicate values according to PCA assignment (Table 2).

GIS results

The diurnal species that we measured were spread across climate zones (Fig. 6A). Although we only measured three known facultative-low-light species, two were primarily collected in the tropics and one in the subtropics (Fig. 6B). Nine of the known obligate-low-light foraging bee species were mostly collected in tropical climates, with three species collected primarily in arid, subtropical and temperate climates, respectively (Fig. 6C). Most facultative-associated bee species were tropical, with some primarily subtropical, arid and temperate species (Fig. 6A, B; Table 2). Climate-zone results for each species are given in Suppl. material 6: Fig. S5 and for each collection event in Suppl. material 1: Table S7. Our Chi-squared and Fisher's Exact tests were non-significant for our pre-PCA behavioural assignments ($\chi^2 = 8.4$, $p = 0.2$; Fisher's $p = 0.2$; Fig. 6), but significant for our post-PCA assignments ($\chi^2 = 13.2$, $p = 0.03$; Fisher's $p = 0.03$; Fig. 6 and Table 2). Both facultative and obligate low-light foragers were more likely to have tropical distributions than expected by chance (Suppl. material 1: Table S8).

Discussion

Bee fauna where foraging behaviour includes, or is restricted to, dim-light conditions is vastly understudied. For example, there are no previously published records of Australian twilight-foraging for any species, although several have been hypothesised to be

low-light adapted. The observations of *Reepenia bituberculata* on the palms *Dypsis lutescens* and *Licuala ramsayi*, and *Meroglossa gemmata* on *Melaleuca leucadendra*, represent the first plant records for these bee species, and the first confirmed crepuscular foraging behaviour for any Australian bee species. The collection of several genera, including three species of *Meroglossa*, on *M. leucadendra* after sunset are important records of the surprising diversity of bees foraging into the evening twilight (Suppl. material 1: Table S1). If researchers restrict collecting times to typical daylight hours an unknown portion of diversity might be missed. Hence, we encourage researchers to widen their sampling times to better survey low-light foraging bee fauna, allowing further studies on the evolution of this behaviour and associated morphological traits. Many of the species collected on *M. leucadendra* in the evening were represented by only a single or a few specimens, and more observations are needed to confirm how frequently these species forage in low-light conditions compared to diurnally, and whether the behaviour is geographically widespread or restricted to northern Australia. Such foraging habits might be expected to vary by climate and season (Wcislo and Tierney 2009); in Australia this is unexplored.

Our PCA analyses were able to cleanly separate diurnal from obligate low-light adapted species. However, they did not sharply separate facultative low-light bees from diurnal species (Fig. 4; Suppl. material 3: Fig. S2, Suppl. material 4: Fig. S3 and Suppl. material 5: Fig. S4). Our discriminant analyses of principal components were able to correctly predict photic niche membership in 100% of cases. The 95% confidence limits of discriminant scores were also able to clearly separate diurnal and low-light behavioural groups (Fig. 4C, D). These results are important for two reasons: firstly, they indicate that principal component and discriminant function score values are very distinct for diurnal and low-light groups; secondly, they suggest that for species where photic niches are not discrete, discriminant function scores are intermediate between those for diurnal and low-light species. Combined, our analyses suggest clear morphological adaptations to photic niches, possibly even for facultative low-light species. However, the inclusion of more known facultative low-light species is required to corroborate the latter results.

Our PCA analyses indicated morphological associations with behavioural groups. Obligate low-light-adapted bees tended to be moderate to large in size, have lower ommatidial densities, larger eye to head ratios and a larger median ocellus width to ocello-ocular distance ratios than diurnal or facultative species. As low-light adapted bees became larger (e.g., *X. tabaniformis* Smith, 1854 and *X. myops* Ritsema, 1876) the latter two factors became smaller; likely because even though median ocellus width and eye size remained large, they were reduced as a ratio (Fig. 4; Suppl. material 3: Fig. S2). Larger bees could be more likely to adapt to low-light conditions because they should have fewer constraints to the development of large eyes (Wcislo and Tierney 2009) and an increased thermoregulation capacity (Hrncir and Maia-Silva 2013; Streinzer et al. 2016). Hence, large body size has been referred to as 'pre-adaptation' to low-light behaviours (Wcislo and Tierney 2009). In general, facultative low-light species were small to moderate in size, had larger eye to head ratios and a larger median ocellus width to

ocello-ocular distance ratios than diurnal species. Additionally, morphological character states that might be beneficial for low-light foraging, but selected against for diurnal behaviours are not likely to be maintained in facultative low-light bees. For example, our PCA analyses indicates that low ommatidial density might be one such character that could be selected against for diurnal activity perhaps due to reduced visual resolution or excess light sensitivity (Fig. 4). Such negatively-selected characters might even encourage the fixation of obligate behaviour from facultative behaviour by directional or disruptive selection; but, this would require further examination to corroborate.

The broad spread of low-light-adapted bee species in our PCA analyses might indicate different morphological strategies adopted by facultative or obligate low-light foraging species (Fig. 4; Suppl. material 3: Fig. S2, Suppl. material 4: Fig. S3 and Suppl. material 5: Fig. S4). There is also a gradient in terms of the extent of low-light morphological traits and behaviours (Fig. 4). Our analyses indicated loose groupings of low-light adapted species by families, but more species need to be measured to thoroughly examine family-level morphological strategies (Fig. 4A).

Our observations and PCA analyses both suggest that facultative low-light behaviour is more common than published reports would indicate (Fig. 4; Table 2; Suppl. material 1: Tables S1, S2, S6). In addition to observations of *R. bituberculata* and *Meroglossa* species, we collected 14 morphospecies that were foraging before sunrise or after sunset (Suppl. material 1: Tables S1, S2). Only *Amegilla cingulata* (Suppl. material 1: Table S6) and *Homalictus atrus* (Suppl. material 1: Table S2) had both observational data of low-light behaviour and also morphological analyses conducted, providing dual support for facultative low-light behaviour in these two species. Two other *Amegilla* species (*A. aeruginosa* and *A. adelaidae*) as well as the kleptoparasite of *A. cingulata* (Houston 2018), *Thyreus nitidulus*, were recovered as facultative-associated species in our PCA analysis. This could indicate a phylogenetically-conserved trait for these *Amegilla* species and ecological association for the kleptoparasitic *T. nitidulus*, although this is not as clear for *T. caeruleopunctatus* (Fig. 4; Suppl. material 3: Fig. S2). Additionally, some *Megachile* (Megachilidae) and *Nomada* (Apidae) species have been suggested to be low-light adapted (Wcislo and Tierney 2009).

Interestingly, *Lasioglossum ochroma*, which has previously been hypothesized to be crepuscular due to its reduced pigmentation (Dorey 2018; Houston 2018), has no confirmed low-light collection records (Walker 1995; ALA 2020) and in our PCA was grouped with diurnal species (Fig. 4). Additionally, the specimen used in this analysis was collected at midday (Dorey 2018). Because many apparently facultative low-light adapted species lack pale integument (e.g., the mostly black *M. gemmata*) and many diurnal species exhibit it (particularly arid-adapted species, e.g., *L. ochroma* and many Euryglossinae: Colletidae species), we suggest that this character need not implicate low-light foraging behaviour, in agreement with the findings of Wcislo and Tierney (2009). However, in the Euryglossinea, pale colouration could be an adaptation to other drivers (e.g. crypsis on flowers or temperature regulation) and not due to a loss of positive selection.

Facultative-associated males, particularly those outside of the tropics or arid regions (see below), should be regarded more critically as visual-adaptations might be

influenced by mating pressures. For example, males in many allodapine species, like *Exoneura* cf. *xanthoclypeata* (Apidae; which grouped roughly with the facultative low-light species), can have enlarged compound eyes but the female clearly grouped with diurnal species (Suppl. material 3: Fig. S2). Although, this alone might not explain the enlarged ocelli in other species, like *E. robusta* Cockerell, 1922 (not included in our analyses). Additionally, collections of *Pharohylaeus lactiferus* (Cockerell, 1910) (Colletidae) were primarily made during daylight hours despite observations extending into early morning and late afternoon (Dorey in press); however, *P. lactiferus* is a rare species and more observations are required.

Our GIS analyses show that most of our facultative low-light species have primarily tropical distributions, but with some species occurring in subtropical, temperate and arid climate-zones (Fig. 6). Most species of obligate low-light adapted bees are thought to be tropical, subtropical or arid (Warrant 2008; Wcislo and Tierney 2009), making the occurrence of *Xylocopa tabaniformis* in temperate regions unusual; however, it occurs where summers are warm or hot (Beck et al. (2018) (Suppl. material 1: Table S7). A disproportionate number of facultative-associated species have primarily tropical distributions with some species occurring in subtropical, arid and temperate climate zones (Fig. 6B). This suggests that tropical areas should be targeted to further examine facultative low-light adapted bee species. A complete phylogeny for these taxa does not exist; however, it is worth noting that phylogenetic relationships between species might break assumptions of independence for Chi-squared analyses. Hence, these results should be interpreted with this caveat in mind. The precise drivers behind the climate-specific distributions of low-light adapted bees is as of yet unknown. The climate-associated factors that might drive low-light-specialisation include: the distribution and phenology of nocturnally-flowering plants, floral specialisation, relative rates of change in light intensity and even learned behaviours (Wcislo and Tierney 2009). It is also possible that escape from extreme daytime temperatures (Wcislo and Tierney 2009) and warm night time temperatures could encourage the evolution of low-light foraging behaviour. We did not attempt to collect or analyse the temperature or light-levels required for flight for any of the species included in the morphometric analyses, but this is another important research avenue that might provide more insight into the patterns of low-light adaptation in bee fauna.

There is little understanding of the importance of low-light and nocturnal foraging bees as pollinators in different environments, and even in studies recording nocturnal or crepuscular bee visitors to flowers, their contribution to pollination is mostly unknown (e.g., Krug et al. (2015)). In some ecosystems, however, low-light foraging bees are critically important; nocturnal bees have recently been established as the only effective pollinators of *Campomanesia phaea* (O. Berg.) (Myrtaceae) in Brazil (Cordeiro et al. 2017), whilst *Heterophragma quadriloculare* (Roxb.) K.Schum. (Bignoniaceae) is reported as being solely pollinated by the nocturnal carpenter bee *Xylocopa tenuiscapa* Westwood, 1840 (Somanathan and Borges 2001). How important crepuscular, vespertine or matinal foraging bees are for the pollination of various plant species and broader ecosystem services is mostly unknown. Evidence suggests that bees foraging in low-light conditions are attracted to flowers by olfactory cues

(Carvalho et al. 2012; Cordeiro et al. 2017) and timing of activity can sometimes be affected by moon phase (Kerfoot 1967b; Roberts 1971). Crepuscular and nocturnal bees visiting guarana (*Paullinia capana*, Sapindaceae) flowers in Brazil commenced activity an hour earlier in the phases of full and waning moons (Krug et al. 2015) but the moon phase had no effect on *X. tenuiscapa* Westwood, 1840 foraging on *H. quadriloculare* in India (Somanathan and Borges 2001). The extent to which moon phase, season and climatic zone (for broadly-distributed species) impacts low-light behaviour still requires further study.

Conclusions

The effort to document global biodiversity continues alongside attempts to monitor geographical and phenological shifts in flora and fauna brought on by climate change. Our observations, collections and PCA analyses confirm the crepuscular behaviour of *R. bituberculata* and *M. gemmata*. We also present behavioural data to support facultative low-light behaviour of *M. eucalypti* and *M. impressifrons penetrata*. Additional observations and collections suggest facultative low-light behaviour of several other Australian bee taxa, but these require further examination.

Many species recorded as foraging in low-light conditions, or caught in light traps, have low-light-associated traits such as enlarged ocelli and enlarged compound eyes. However, behaviour is difficult to determine for many species, particularly those that are facultative low-light foragers rather than obligate (Wcislo and Tierney 2009). Our analyses indicate that it is possible to separate diurnal from obligately low-light-adapted bee species using morphological characters. In contrast to Wcislo and Tierney (2009), we show that facultative low-light-adapted bees do exhibit a suite of morphological characters that appear to differentiate them from diurnal species; however, additional data and behavioural studies are needed to corroborate our results. Thus, we provide an analytical framework to aid in the morphological identification of low-light-adapted bees that is otherwise lacking. We further provide the data that is needed to put other species into this context and infer their behavioural state. This is important as, according to our observations and analyses, low-light foraging behaviour appears to be far more common than previously appreciated. However, collections of actively-foraging bees remains critical to determining the extent of low-light activity, particularly for facultative low-light foraging species. The addition of these data will help to confirm and perhaps expand the behavioural assignments implied by our PCA and DAPC analyses. Additionally, the apparent rarity of low-light foraging species might be explained by the diurnal activities of mellitologists. Hence, placing such species in this analytical framework could aid in their collection and further study.

Globally, weather patterns are changing (IPCC 2014) and in many parts of Australia, temperatures are rising and droughts and fires are becoming more prolonged (CSIRO and Bureau of Meteorology 2015). Hence, the importance of understanding the ecological roles of understudied taxa like nocturnal bees and their climatic niches is

becoming even more imperative. Documenting the insects that are providing pollination services during milder parts of the day might provide insights into understanding potential adaptation in the future. Conversely, we need to highlight the species with narrow foraging windows, which might be sensitive to change and therefore require conservation attention. There is a need to increase efforts into collecting outside of 'normal' diurnal hours, recording times of collection with specimens, and publishing observations of low-light foraging behaviour. This will further our ability to tackle a broad spectrum of questions on the conservation, behaviour, physiology and evolution of bees and the role that they play in maintaining ecosystem services.

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Supplementary material 1

Tables S1–S8

Authors: James B. Dorey, Erinn P. Fagan-Jeffries, Michael P. Schwarz

Data type: Scree plots and heat maps of factor loadings from PCA analyses

Explanation note: **Table S1.** Collection information for specimens. Specimens with a sample ID (SAMA number) are deposited at the South Australian Museum. **Table S2.** Data used to construct Fig. 1 observations and collections of *Reepenia bituberculata* and *Meroglossa* spp. The recorded observations of these species were undertaken during a waning gibbous (Daintree and Laura observations) and waning crescent (Cairns observations) moons. **Table S3.** The species, species authority, family, specimen code, sex, behavioural state (white; diurnal, yellow; facultative and blue; low-light), published reference for behavioural state, climate-zone and Morphological measurements used in the PCA analyses. **Table S4.** The standard deviation, proportion of variance and cumulative proportion of variance for all principal components from our PCA analyses. Analyses are separated by sex. **Table S5.** Standardized canonical discriminant function coefficients for both female and male discriminant analyses. **Table S6.** Observations of roosting *Amegilla cingulata* in subtropical Queensland, Australia. Observations were made late in the season (i.e. when temperatures were relatively cool) and were undertaken by citizen scientist, Jenny Thynne. **Table S7.** The basis of record, event date and time, political state, latitude, longitude, species and climatic state of all specimens that were accessed from ALA and GBIF and included in our GIS analysis. **Table S8.** The observed and expected number of species by behaviour and climate zone for our Chi-Squared analyses both before (p = 0.2) and after (p = 0.03) PCA behavioural assignments.

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Link: https://doi.org/10.3897/jhr.79.57308.suppl1

Supplementary material 2

Figure S1
Authors: James B. Dorey
Data type: Scree plots and heat maps of factor loadings from PCA analyses
Explanation note: Scree plots of principal components (proportion of variance; top) and heatmaps and values of the loadings of measurements on each principal component (bottom) for females **A** and males **B**.
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Link: https://doi.org/10.3897/jhr.79.57308.suppl2

Supplementary material 3

Figure S2
Authors: James B. Dorey
Data type: Figure of PCA plots
Explanation note: Principal components one and two of female **A** and male **B** and principal components three and four of female **C** and male **D** bees where labels indicate species and colour indicates known foraging behaviour (diurnal: red, facultative: green and low-light: red). Measurements are defined in Fig. 1. These plots indicate relative factor loadings for the morphological traits as vectors from the centroid.
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Link: https://doi.org/10.3897/jhr.79.57308.suppl3

Supplementary material 4

Figure S3

Authors: James B. Dorey

Data type: Interactive html 3D figure – to be opened by a web browser

Explanation note: Principal components one, two and three for female bees where colour indicates known foraging behaviour of bees (diurnal: red, facultative low-light: green and obligate low-light: red). Measurements are defined in Fig. 1. Relative factor loadings are indicated for the morphological traits as vectors from the centroid.

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Link: https://doi.org/10.3897/jhr.79.57308.suppl4

Supplementary material 5

Figure S4

Authors: James B. Dorey

Data type: Interactive 3D figure – to be opened in a web browser

Explanation note: Principal components one, two and three for male bees where colour indicates known foraging behaviour of bees (diurnal: red, facultative low-light: green and obligate low-light: red). Measurements are defined in Fig. 1. Relative factor loadings are indicated for the morphological traits as vectors from the centroid.

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Link: https://doi.org/10.3897/jhr.79.57308.suppl5

Supplementary material 6

Figure S5

Authors: James B. Dorey

Data type: PDF book of climate frequency plots of specific bee species

Explanation note: Frequency plots of identified bee species, ordered by family, for each climate zone. Plots with no bars had no reliable coordinates.

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Link: https://doi.org/10.3897/jhr.79.57308.suppl6